Genetic diversity of *Mycoplasma hyopneumoniae* isolates of abattoir pigs

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Abstract

Mycoplasma hyopneumoniae, the causative agent of porcine enzootic pneumonia, is present in swine herds worldwide. However, there is little information on strains infecting herds in Canada. A total of 160 swine lungs with lesions suggestive of enzootic pneumonia originating from 48 different farms were recovered from two slaughterhouses and submitted for gross pathology. The pneumonic lesion scores ranged from 2 to 84%. Eighty nine percent of the lungs (143/160) were positive for *M. hyopneumoniae* by real-time PCR whereas ten percent (16/160) and 8.8% (14/160) were positive by PCR for *M. hyorhinis* and *M. flocculare*, respectively. By culture, only 6 % of the samples were positive for *M. hyopneumoniae* (10/160). Among the selected *M. hyopneumoniae*-positive lungs (*n*=25), 9 lungs were co-infected with *M. hyorhinis*, 9 lungs with PCV2, 2 lungs with PRRSV, 12 lungs with *S. suis* and 10 lungs with *P. multocida*. MLVA and PCR-RFLP clustering of *M. hyopneumoniae* revealed that analysed strains were distributed among three and five clusters respectively, regardless of severity of lesions, indicating that no cluster is associated with virulence. However, strains missing a specific MLVA locus showed significantly less severe lesions and lower numbers of bacteria. MLVA and PCR-RFLP analyses also showed a high diversity among field isolates of *M. hyopneumoniae* with a greater homogeneity within the same herd. Almost half of the field isolates presented less than 55% homology with selected vaccine and reference strains.

Keywords: *Mycoplasma hyopneumonia*e, porcine enzootic pneumonia, genotyping, MLVA, PCR-RFLP

Introduction

Mycoplasma hyopneumoniae is present in the majority of swine herds around the world (Kobisch and Friis, 1996). It is the primary agent involved in porcine enzootic pneumonia (EP). This condition is associated with respiratory disease and reduced productivity in pigs causing severe economic losses to the swine industry. The importance of *M. hyopneumoniae* is also linked to its ability to increase the severity of infections caused by viruses (Opriessnig et al., 2004), as well as bacteria (Marois et al., 2009; Palzer et al., 2008). When these pathogens are in co-infection with *M. hyopneumoniae*, the severity of the respiratory lesions is increased. Moreover, *M. hyopneumoniae* can enhance the quantity and the persistence of PVC2 antigens and can increase the incidence of postweaning multisystemic wasting syndrome (PMWS) in swine (Opriessnig et al., 2004; Thacker et al., 2001).

Isolation of *M. hyopneumoniae* is known to be fastidious due to the long incubation period needed for its culture (Friis, 1975; Marois *et al*., 2007) and to the frequent co-isolation of *Mycoplasma hyorhinis*, a normal flora inhabitant of the upper respiratory tract of young pigs (Kobisch and Friis, 1996). *M. hyorhinis* has also been involved in a variety of diseases in swine including enzootic pneumonia and respiratory disease in general (Kawashima et al., 1996; Kobisch and Friis, 1996; Lin et al., 2006).

At the genomic level, high heterogeneity has been demonstrated between *M. hyopneumoniae* isolates throughout the world using various typing techniques such as random amplified polymorphic DNA (RAPD) (Artiushin and Minion, 1996), amplified

fragment length polymorphism (AFLP) (Kokotovic *et al*., 1999) and pulsed-field gel electrophoresis (PFGE) (Stakenborg et al., 2005). However, the RAPD technique and the analysis of polyserine repeats have weak reproducibility rates among different laboratories, and the AFLP and PFGE techniques are considered fastidious. Thus, new techniques based on DNA amplification have been developed in the last few years. The multiple loci variable number of tandem repeats (VNTR) analysis (MLVA) and the PCR combined with restricted fragments length polymorphism (PCR-RFLP) are two methods that can be easily performed, are reproducible and have a high discriminatory power (Marois-Créhan *et al*., 2012; Stakenborg *et al*., 2006b; Vranckx *et al*., 2011). Recently, a MLVA assay was described as a tool to differentiate *M. hyopneumoniae* strains in samples from the respiratory tract without prior cultivation (Vranckx *et al*., 2011). Previous studies have shown genetic heterogeneity between isolates from different farms (Mayor et al., 2007; Nathues et al., 2011; Stakenborg et al., 2005). However, other reports have shown both genetic heterogeneity and homogeneity between isolates from the same herds (Maes *et al*., 2008; Marois-Créhan *et al*., 2012). Field isolates of *M. hyopneumoniae* have also shown virulence variability (Vicca *et al*., 2003).

Actually, little is known about *M. hyopneumoniae* isolates found in Canada. The aim of this study was to evaluate the genetic diversity of *M. hyopneumoniae* isolated from single or mixed infections from abattoir pigs.

Materials and Methods

Sample collection and histopathology

A total of 160 swine lungs presenting gross lesions suggestive of porcine enzootic pneumonia, originating from 48 farms, were recovered from two slaughterhouses (#1, *n*=110; #2, *n*=50) located in the province of Quebec (Canada) from October 2008 to March 2009. The lungs were all scored for macroscopic pneumonic lesions as previously described by Straw *et al.* (Straw, 1986). For *M. hyopneumoniae* isolation, swabs from the trachea and lungs were resuspended in 1 mL of buffered peptone water. A subset of 25 *M. hyopneumoniae*-positive lungs by real-time PCR (Table 1) were further analysed for the detection of *Streptococcus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae* using traditional bacteriology methods, PRRSV (Real-time PCR diagnostic test kit, Tetracore, Rockville, Maryland, USA) and PCV2 (Gagnon *et al*., 2008) (Virology laboratory, Faculty of Veterinary Medicine, University of Montreal). A portion of each affected lobe was fixed in 10% neutral buffered formalin for histopathology examinations. A minimum of four and a maximum of five sections were embedded in paraffin, cut at 5µm, and stained with hematoxylin, eosin, phloxin B and saffron for light microscopic examination.

M. hyopneumoniae isolation

For each sample, one hundred µL of the initial buffered peptone water suspension was inoculated in 900 µL of Friis medium supplemented with bacitracin (150 µg/mL), amphotericin B (2.5 µg/mL), ampicillin (100 µg/mL) and colistin (7.5 µg/mL) (all from

Sigma, Oakville, Ontario, Canada) to optimise *M. hyopneumoniae* recovery (Marois *et al*., 2007). Tubes were incubated at 37°C until the culture developed an acid colour change or up to 30 days. The cultures were then ten-fold serially diluted up to 10^{-3} (Marois et al., 2007) and 10 µl of each dilution was plated onto NHS agar for purification (Friis, 1971). Plates were incubated at 37°C with 5% CO_2 for 1-2 weeks. Isolated colonies were recovered, inoculated in 1 mL of Friis medium and incubated until color shift.

Real-time PCR

Initial buffered peptone water suspensions were examined for the presence and the quantification of *M. hyopneumoniae* by real-time PCR. Primers were used as described by Strait *et al.* (Strait *et al*., 2008). Briefly, DNA extraction was performed with the QIAamp DNA Mini Kit (Qiagen, Mississauga, Ontario, Canada). The Quantitect Probe PCR kit (Qiagen) was used for the reaction. The PCR mix contained 1X PCR buffer, 400 nM of each primer, 120 nM of TaqMan probe, 5 µL of DNA and completed to 25 µL with sterile water without RNAse. DNA amplification was carried out using a Cepheid SmartCycler® system (Fisher Scientific, Ottawa, Canada) with the following conditions: 15 min at 95°C then 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. *M. hyopneumoniae* ATCC 25095 was used as a positive control. For the quantification, Ct values of the samples were compared to the standard curve. The standard curve was elaborated with serial dilutions of known concentrations of DNA from *M. hyopneumoniae* ATCC 25095. The detection limit of this technique was 1000 genomes/mL.

Conventional PCR

Shifted cultures were examined for the presence of *M. hyopneumoniae, M. hyorhinis* and *Mycoplasma flocculare* in a multiplex PCR. Initial buffered peptone water suspensions previously analysed by real-time PCR for *M. hyopneumoniae* were also examined for the presence of *M. hyorhinis* and *M. flocculare* in a multiplex PCR. Primers were as described by Stakenborg *et al.* (Stakenborg *et al*., 2006a). Briefly, DNA extraction was performed as described above following the "DNA purification from tissues" protocol. The PCR mixture contained 1X PCR buffer, 200 µM of dNTPs, 160 nM of each forward primer, 240 nM of the reverse primer, 1 unit of Taq DNA polymerase (New England Biolabs, Whitby, Ontario, Canada), and 5 µL of the DNA template in a total volume of 50 µL. Amplification was performed in a Whatman Biometra thermocycler (Montreal Biotech Inc, Montréal, Quebec, Canada). The reaction procedure consisted of 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 54.6°C for 15 sec, and extension at 68°C for 1 min. A volume of 10 µL of each PCR product was separated for 30 min at 150 V on a 1.5 % agarose gel stained with ethidium bromide. *M. hyopneumoniae* ATCC 25095, *M. hyorhinis* ATCC 17981 and *M. flocculare* ATCC 27399 were used as positive controls.

MLVA and PCR-RFLP

For genotyping of *M. hyopneumoniae*, four loci with a VNTR were used (Table 2). DNA extracts were from initial buffered peptone water suspensions from lungs or from isolates. The PCR mixture for the four loci contained 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM of dNTPs, 400 nM of each primer (Table 1), 1 units of Taq DNA polymerase (New England

Biolabs), and 5 µL of the DNA template in a total volume of 50 µL. Amplification was performed in a Whatman Biometra thermocycler. The reaction procedure for loci P97-RR1 and P97-RR2 consisted of 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 54°C for 30 sec, and extension at 72°C for 1 min. The reaction procedure for loci 1 and 2 consisted of 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Ten µL of each PCR product was run for 40 min at 125 V on a 2% agarose gel which was stained with ethidium bromide for 30 min. PCR-RFLP analysis was performed as described by Stakenborg *et al.* (Stakenborg *et al*., 2006b) with few modification. Briefly, the p146 gene was amplified in the following conditions: 1x PCR buffer, 2 mM $MgCl_2$, 0.1 mM of dNTPs, 20 pmol of both primers (Table 2) and 2.5 U of Taq DNA polymerase (New England Biolabs). The amplification reaction was 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 52.5°C for 30 sec, and extension at 68°C for 3 min followed by 15 min at 68^{o}C. Seventeen µL of the final PCR product was digested for 3 h at 37^{o}C in a mix containing 10 U of restriction enzyme *AluI* (New England Biolabs). Restricted fragments were separated for 60 min at 120 V on a 2% agarose gel which was then stained with ethidium bromide for 30 min.

M. hyopneumoniae ATCC 25934, ATCC 25095 and strain 232 were used as a positive controls. Strains from France (n=10), DNA extracts from 6 vaccines available in North America and two lungs with lesions suggestive of EP from Manitoba were also included in genotyping analysis for comparison purposes.

Cluster and data analysis

Digital images of PCR-RFLP gels and MLVA profiles, created from the number of repeats for each of the VNTR loci, were imported to the BioNumerics software (Applied Maths, Austin, TX, USA). The unweighted pair group method with arithmetic mean (UPGMA) was used for clustering. For PCR-RFLP, bands were marked following standardisation using the TrackIt 100 pb DNA ladder (Life Technologies, Burlington, Ontario, Canada). Band position tolerance and optimisation were set to 1%. Bands smaller than 175 pb were omitted (Stakenborg *et al*., 2006b). The discriminatory power of the MLVA and PCR-RFLP techniques were calculated by using the Simpson's index of diversity. Two different indexes were calculated for each technique, one including all *M. hyopneumoniae* isolates and one excluding all isolates demonstrating an identical fingerprint and originating from a single herd. An exact multivariate logistic regression was used to determine the relationships between *M. hyopneumoniae* and other pathogens found in co-infections. Student "t" test was performed to evaluate the relationship between the absence of amplification of locus 1 and the lower concentration of bacteria and percentage of lesions. All statistics were done with the SAS software v.9.1. (Cary, N.C.). A $p< 0.05$ was considered to be significant.

Results

Pathology and Histopathology

All lungs with lesions suggestive of EP (*n*=160) were submitted for gross pathology. The macroscopic pneumonic lesion scores ranged from 2 to 84% and these were confirmed by histopathological examination. Mild post-mortem changes were present in all samples, although preservation of tissues was adequate. Lymphoid hyperplasia of the bronchus-associated lymphoid tissue (BALT) was moderate to marked, except in 3 pigs for which it was mild. Evidence of compression of bronchioles by lymphoid nodules/follicles was sometimes present. In all but one pig, hyperplasia was associated with suppurative bronchitis/bronchiolitis or bronchopneumonia, mucus being admixed with the neutrophils in the bronchi of a few pigs. Intra-alveolar edema and macrophages were observed multifocally, the number of macrophages being moderate to high in 14/25 pigs, and low in the others. Bronchiolitis obliterans was observed in 22/25 pigs, usually not marked or extensive except in 3 pigs where it was prominent. Finally, in 9/25 pigs, a few to several small alveolar and/or bronchiolar granulomata associated with plant material were present.

PCR analysis

A total of 89.3 % of the swabs from the tested lungs with lesions suggestive of EP (143/160) were positive by real-time PCR for *M. hyopneumoniae* with a concentration of bacteria ranging from 1.17×10^5 to 3.37×10^9 genomes per mL. No link was observed between numbers of *M. hyopneumoniae* cells and severity of lesions in abattoir pigs (Fig. S1-A). Using multiplex PCR, 10% of the lungs with lesions suggestive of EP (16/160)

were positive for *M. hyorhinis* whereas 8.8% (14/160) were positive for *M. flocculare*. Two lungs (1.25%) carried a mixture of the three mycoplasmas whereas no mycoplasmas were detected in seven lungs (4.4%). None were positive for *M. flocculare* without the other two mycoplasmas while seven lungs (4.4%) were positive for *M. hyorhinis* alone.

Bacterial isolation

Only 10 isolates of *M. hyopneumoniae* were recovered out of the 160 lungs cultured. Of those, 9 mixed cultures of both *M. hyopneumoniae* and *M. hyorhinis* were observed. *M. hyopneumoniae* was recovered in pure culture in only one lung. No *M. flocculare* was recovered whereas two pure cultures of *M. hyorhinis* were isolated from two different lungs. All positive broth cultures (*n*=12) were tested by multiplex PCR for the identification of *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (Table 1). The overall isolation rate for both slaughterhouses was 6.2% for *M. hyopneumoniae* and 6.9% for *M. hyorhinis*. Interestingly, the recovered isolates were all from slaughterhouse #2 (*n*=12/50) indicating an isolation rate for this unit of 20% for *M. hyopneumoniae* (*n*=10/50) and of 22% for *M. hyorhinis* (*n*=11/50).

Co-infection

A subset of positive lungs (*n*=25) were selected for the co-infection study. The first criteria of selection was positivity on culture for *M. hyopneumoniae* and/or *M. hyohinis* (Table 1) which yield only 12 positive lungs. Because of the low number of isolates recovered, an additional 13 lungs were randomly selected based on real-time PCR results

to complete the subset (Table 1). Of those selected lungs (*n*=25), nine were co-infected with *M. hyorhinis*, nine with PCV2 and two with PRRSV as determined by PCR (Table 1). Using traditional bacteriology culture, 12 lungs were found to be co-infected with *S. suis* and 10 lungs with *P. multocida* whereas no co-infection was observed with *H. parasuis*, *A. pleuropneumoniae* and *A. suis* (Table 1). The odds of being in the group with more severe lesions were not associated with the prevalence of *M. hyorhinis* (p = 0.45), VSRRP (p = 0.23), CVP2 (p = 0.15), *S. suis* (p = 0.20) and *P. multocida* (p = 0.22).

MLVA typing

MLVAs were performed directly on all *M. hyopneumoniae*-positive lungs from Quebec (*n*=143) and Manitoba (*n*=2), broth cultures (*n*=10), vaccines (*n*=6) and reference (*n*=3) and France (*n*=10) strains. Results obtained from cultured broths and their associated lungs were identical. A total of 87 MLVA types were identified and these types were further separated in five clusters, the cluster one and three containing the majority of the isolates (Fig. 1). The first cluster contained all three reference strains and all vaccine strains. The Simpson's index of diversity was 0.997, indicating a high discrimination power, when all isolates with identical fingerprints and originating from the same farm were excluded. This index was 0.991, indicating a slightly lower discrimination power, when all *M. hyopneumoniae* isolates were taken onto account. All isolates were considered typable by MLVA because in all cases at least one locus out of four was amplified. Fifteen isolates had no amplification for one of the four loci (locus one, *n*=14; and locus P97-RR1, *n*=1), one isolate for two loci (P97-RR1 and P97-RR2) and one isolate for three loci (locus one, P97-RR1 and P97-RR2). MLVA clustering of *M.*

hyopneumoniae from abattoir pigs revealed that analysed strains were distributed among the five clusters regardless of lesions' severity, indicating that no MLVA clusters were associated with virulent strains (Fig. S1-B). However, strains lacking locus 1 showed significantly less severe lesions and lower numbers of bacteria as demonstrated by histopathology and real-time PCR, respectively ($p < 0.05$). These isolates were from cluster 3 (n=14) or cluster 5 (n=1). Their corresponding lungs presented between 10^2 to 10^4 times less bacteria (data not shown). Also, thirteen lungs showed amplification of more than one band indicating that they harbor more than one isolates. MLVA revealed no amplification for *M. hyorhinis*, *M. flocculare*, *S. suis*, *H. parasuis*, *P. multocida*, *A. suis* or *A. pleuropneumoniae*.

PCR-RFLP analysis of the p146 encoding gene

PCR-RFLPs were also performed directly on all *M. hyopneumoniae*-positive lungs from Quebec (n=143) and Manitoba (n=2), broth cultures (n=10), vaccines (n=6), reference (n=3) and France (n=10) strains. Again, results obtained from cultured broths and their associated lungs were indistinguishable. For this procedure, forty-one lungs, one strain from France and the vaccine strains were non-typeable because of the absence of amplification of the p146 gene. The remaining *M. hyopneumoniae*-positive lungs (n=102) were separated into 83 different patterns and regrouped in seven clusters (Fig. 2). The second cluster contained all reference strains. The Simpson's index of diversity was 0.986, which is considered an average discrimination rate, when isolates with the same fingerprint and isolated from the same farm were excluded. When all *M. hyopneumoniae* isolates were taken into account, the index was 0.971. There was no association between

lesions' severity and PCR-RFLP clustering of *M. hyopneumoniae* from abattoir pigs (Fig. S1C). Cluster 3 contained only strains from France but data on lung lesions for these strains were not available (Fig. S1C). No amplification for *M. hyorhinis, M. flocculare, S. suis, H. parasuis*, *P. multocida*, *A. suis* or *A. pleuropneumoniae* were observed in PCR-RFLP.

Discussion

Molecular typing methods are valuable tools to differentiate strains for epidemiological investigations. These techniques become even more valuable when they can be performed directly on clinical samples and for microorganisms that are known to be fastidious. So far, three typing methods not requiring cultivation have been described for *M. hyopneumoniae*: MLVA (Vranckx *et al*., 2011), molecular typing of the p146 gene (sequencing and length of VNTR) (Mayor *et al*., 2007) and multilocus sequence typing (MLST) (Mayor *et al*., 2008). These studies have observed that patterns obtained from cultured broths and their associated clinical samples were identical, indicating that these techniques could be used without prior cultivation. This study also demonstrates that both MLVA and PCR-RFLP should be considered as reliable tests for quick and rather inexpensive differentiation of *M. hyopneumoniae* strains without prior isolation. In addition, MLVA typing gave strong evidence that some pigs were infected with multiple strains of *M. hyopneumoniae* and this was not achieved with the other typing method (Marois-Créhan *et al*., 2012; Vranckx *et al*., 2011). It has been suggested that simultaneous or subsequent infections with more than one strain might result in more severe lung lesions (Villarreal *et al*., 2009; Vranckx *et al*., 2011). However, in this study, lungs harboring more than one strain of *M. hyopneumoniae* as per MLVA results were not associated with higher lesion scores (p=0.87), indicating that numbers of strains per lungs were not linked with more severe lung lesions in abattoir pigs (data not shown). The MLVA procedure also revealed that the absence of one locus was significantly associated to lower concentrations of bacteria (p<0.0001) and lower percentages of lesions (p<0.0001), suggesting that this locus of *M. hyopneumoniae* could be associated

with virulence. The gene amplified by this locus encodes for a hypothetical protein of ~77-78 kDa (GenBank accessions no. YP 278837.1 of strains J (ATCC 25934), YP 115552.1 of USA 232 and YP 287436.1 of 7448). In a study by Calus *et al*. (Calus *et al*., 2007), when comparing the SDS-PAGE patterns from isolates of different herds, the most explicit variability was detected over 74 kDa with one band of ~ 181 kDa specific for two highly virulent isolates. However, attempts to relate total protein profiles according to virulence has been unfruitful (Calus *et al*., 2007). MLVA and PCR-RFLP clustering of *M. hyopneumoniae* also revealed that analysed strains were distributed among all clusters regardless of lesions' severities, indicating that DNA patterns did not cluster according to virulence. Also, no links were observed between numbers of *M. hyopneumoniae* cells and severity of lesions.

MLVA and PCR-RFLP also revealed differences in diversity of *M. hyopneumoniae* strains between farms. This is in accordance with other studies reporting on heterogeneity between herds (Mayor et al., 2007; Nathues et al., 2011; Stakenborg et al., 2005). Additionally, heterogeneity was also observed in *M. hyopneumoniae* found with or without co-infected microorganisms. When isolates from the same farm were compared, the outcome depended on the farm, demonstrating that some isolates were different whereas others identical. These results were confirmed by the Simpson indexes of diversity. These findings have previously been reported (Maes *et al*., 2008) and it is believed that the all-in/all-out production system results in a more uniform population of bacteria compared to the traditional production system which leads to a more heterogeneous group of isolates. Also, the proximity of other farms could contribute to

the introduction of new strains of *M. hyopneumoniae* in a farm, resulting in increase heterogeneity among isolates. The airborne transmission seems to be a mechanism of re-infection for *M. hyopneumoniae* and may occur between farms being up to 9.2 km apart (Dee et al., 2009; Kobisch, 2007; Otake et al., 2010). In this study, some strains were not typeable by PCR-RFLP comparatively to a previous report by Stakenborg *et al*. (Stakenborg *et al*., 2006b). Both studies used different primers. Alignment analysis of the primers of this study with nine known DNA sequences (J, 232, 7448, 168, 168-L, 7422, PMS, BQ14, F7.2C) revealed only conserved regions of the p146 gene. This indicates that mutations are likely responsible for the non-typeable results observed by PCR-RFLP.

This study also reports on molecular variabilities between field and vaccine strains as many *M. hyopneumoniae* (45%) demonstrated less than 55 % homology with the vaccinal and reference strains. Beneficial effects conferred by current commercial vaccines have been previously reported as variable among infected herds. A possible explanation could be the high heterogeneity demonstrated between *M. hyopneumoniae* isolates throughout the world (Artiushin and Minion, 1996; Frey et al., 1992; Kokotovic et al., 1999; Stakenborg et al., 2005). However, this hypothesis warrants further investigations.

Reports on co-infections of PRRS, PCV2 and *P. multocida* with *M. hyopneumoniae* have been associated with increased respiratory lesions and pneumonia severities (Ciprian *et al*., 1988; Opriessnig *et al*., 2004; Palzer *et al*., 2008; Thacker *et al*., 1999; Thacker *et al*., 2001). This has been mainly demonstrated in pigs of 4 to 11 weeks of age. PCV2 infections are most commonly found in pigs between 4 to 8 weeks of age; whereas

PRRSV infections are mostly encountered between 5 to 12 weeks of age (Cho and Dee, 2006; Harding, 1998). In this study, abattoir pigs of approximately 8 months of age were investigated to determine whether these associations were still observable through time. Results showed no significant associations between increased respiratory lesions and the presence of other pathogens in abattoir pigs. Also, the isolation of *M. hyorhinis* alone from lungs with EP has been reported once in Taiwan (Lin *et al*., 2006) but not, to our knowledge, in Canada.

In conclusion, this study showed that *M. hyopneumoniae* isolates differ between herds and, in some cases, even within the same herd in Canada. Moreover, almost half of the field isolates presented less than 55% homology with selected vaccine and reference strains. Isolates did not group according to virulence but the absence of a specific MLVA locus was significantly associated with less severe lesions and lower numbers of bacteria.

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488 **Table 1**: Severity of lesions, quantification of *M. hyopneumoniae* in lungs with lesions

489 suggestive of EP with or without other pathogens in abattoir pigs.

490

Lung identification number	Severity of lesions (%)	*M. hyopneumoniae* culture [a]	*M. hyopneumoniae* quantification (genome/mL)	*M. hyorhinis*	PRRSV	PCV 2	*S. suis*	*P. multucida*	*H. parasuis*	*APP*	*A. suis*
#101	69	*Mhp/Mhr*	$9.20x10^6$	+	-	-	+	+	-	-	-
#105	60	*Mhp/Mhr*	$1.20x10^9$	+	+	-	-	-	-	-	-
#112	57	*Mhp/Mhr*	$4.16x10^8$	+	-	-	+	+	-	-	-
#122	41	*Mhp*	$3.01x10^8$	-	-	-	-	+	-	-	-
#119	20	*Mhr*	$1.14x10^6$	-	-	-	+	+	-	-	-
#120	5	*Mhr*	$1.41x10^6$	-	-	-	+	-	-	-	-
#123	17	*Mhp/Mhr*	$3.25x10^7$	+	-	-	+	+	-	-	-
#125	24	*Mhp/Mhr*	$1.59x10^9$	+	-	-	+	-	-	-	-
#127	23	*Mhp/Mhr*	$9.87x10^7$	+	-	-	-	+	-	-	-
#132	14	*Mhp/Mhr*	$1.90x10^8$	+	-	+	+	-	-	-	-
#135	22	*Mhp/Mhr*	$9.45x10^8$	+	-	+	-	-	-	-	-
#149	17	*Mhp/Mhr*	$5.49x10^8$	+	-	+	-	+	-	-	-
#007	14	-	$3.01x10^7$	-	-	-	-	-	-	-	-
#014	44	-	$1.82x10^6$	-	-	-	-	-	-	-	-
#021	25	-	$6.74x10^7$	-	-	+	-	-	-	-	-
#028	33	-	$3.16x10^8$	-	-	+	+	+	-	-	-
#035	22	-	$1.26x10^7$	-	-	-	+	-	-	-	-
#042	27	-	$5.09x10^7$	-	-	+	-	-	-	-	-
#049	22	-	$1.02x10^8$	-	-	-	+	-	-	-	-
#056	45	-	$1.03x10^8$	-	+	+	-	+	-	-	-
#063	8	-	$4.40x10^8$	-	-	-	-	-	-	-	-
#070	42	-	$4.62x10^8$	-	-	+	-	+	-	-	-
#077	25	-	$2.02x10^6$	-	-	+	+	-	-	-	-
#084	62	-	$1.12x10^8$	-	-	-	-	-	-	-	-
#091	48	-	$2.10x10^8$	-	-	-	+	-	-	-	-

491

492 Results for *M. hyorhinis*, PRRSV and PCV2 are from PCR testing whereas those for *S.*

493 *suis*, *H. parasuis*, *P. multocida*, *A. suis* and *A. pleuropneumoniae* are from traditional

494 bacteriological culture.

495 [a] *Mhp*: *M. hyopneumoniae; Mhr*: *M. hyorhinis;* shifted cultures were confirmed by

496 multiplex PCR

497 PRRSV : Porcine reproductive and respiratory syndrome virus; PCV2 : Porcine

498 circovirus type 2; *APP* : *Actinobacillus pleuropneumoniae*

499 **Table 2:** Primers used in this study for typing by MLVA and PCR-RFLP.
500

PCR	Primers	Sequence (5`-3`)	Products Length (bp)*	Size of repeat (bp)*	Reference
MLVA	Locus1-F	AAACTCCAAAAAAATCGATAAAAA	235	18	(Marois-Créhan et al., 2012)
	Locus1-R	CTTTTTTTGCGGTAATAAGGTTAT			
	Locus2-F	TTTGTGCTTGGTAAAGTTAAATT	108	12	
	Locus2-R	AGAGATTACAACCAAGAAGCAA			
	P97-RR1-F	GAAGCTATCAAAAAAGGGGAAACTA	290	15	(Stakenborg *et al*., 2006b)
	P97-RR1-R	GGTTTATTTGTAAGTGAAAAGCCAG			
	P97-RR2-F	AGCGAGTATGAAGAACAAGAA	344	30	
	P97-RR2-R	TTTTTACCTAAGTCAGGAAGG			
RFLP	P146-F	GCTTTCCATGTTTGGCATTT	2724		This study
	P146-R	CCACAGACAAGTTCTTCGCA			

501

502 * *In silico* estimation from the *Mycoplasma hyopneumoniae* strain J

503 (http://insilico.ehu.es/PCR/)

504

Figure 1: Dendrogram of *M. hyopneumoniae* isolates compared to the vaccine and reference strains as demonstrated by MLVA. Cluster analysis was performed with UPGMA using Pearson coefficient. Reference strains are indicated as broken lines whereas vaccine strains are marked as stars. Tolerance level of 1%. The number of clusters was determined with a 60% similarity cutoff value.

Figure 2: PCR-RFLP dendrogram of *M. hyopneumoniae* isolates compared to the reference strains. Cluster analysis was performed with UPGMA using Dice coefficient. Reference strains are indicated as broken lines. Vaccine strains were not typeable. Tolerance level of 1%. The number of clusters was determined with a 50% similarity cutoff value.

Figure S1: Associations between severity of lesions and numbers of *M. hyopneumoniae* (A), the MLVA types (B) or the PCR-RFLP types (C). Each dot represents *M. hyopneumoniae* from a lung sample. Samples from France were not included because severity scorings were not available.

Figure

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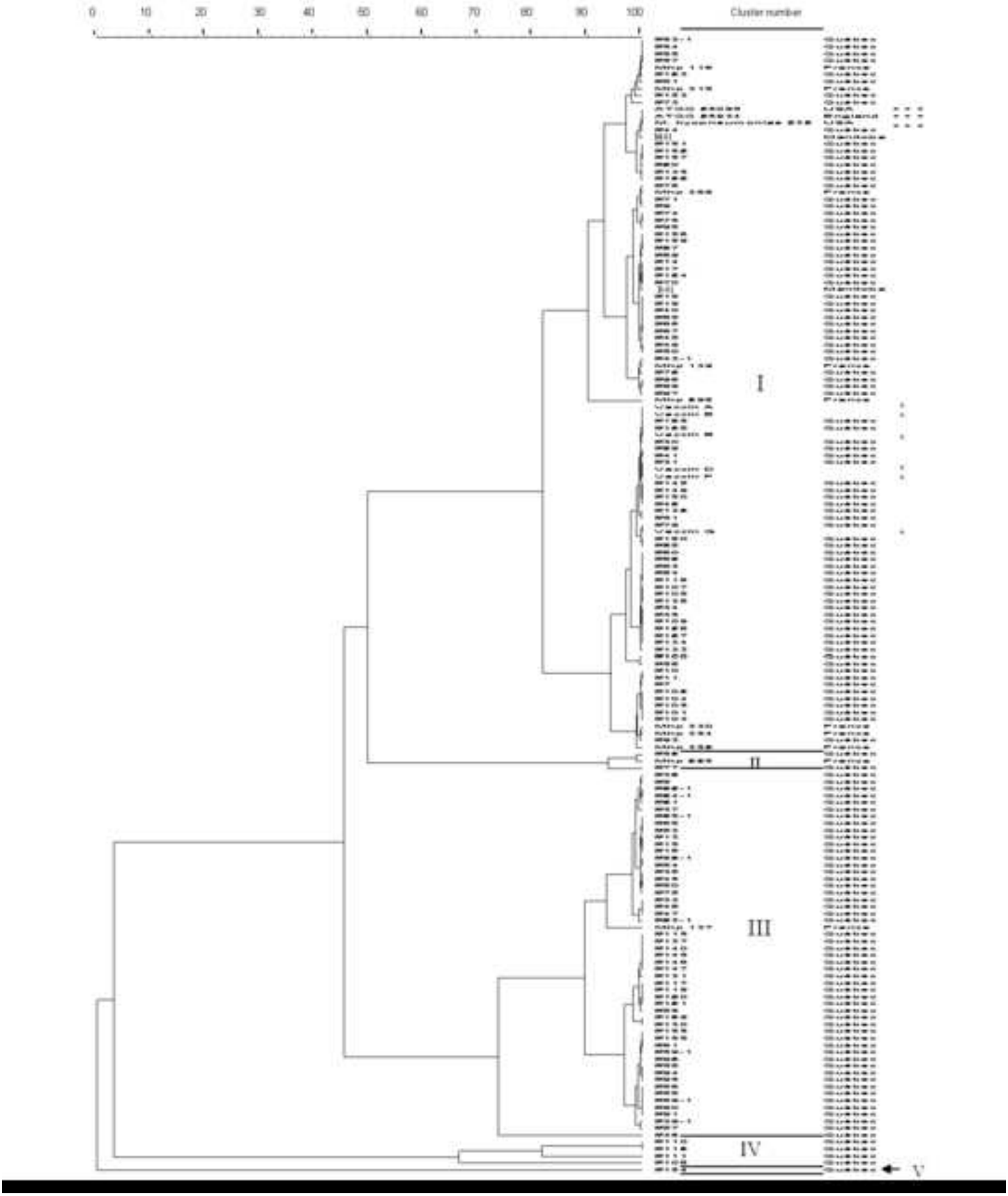

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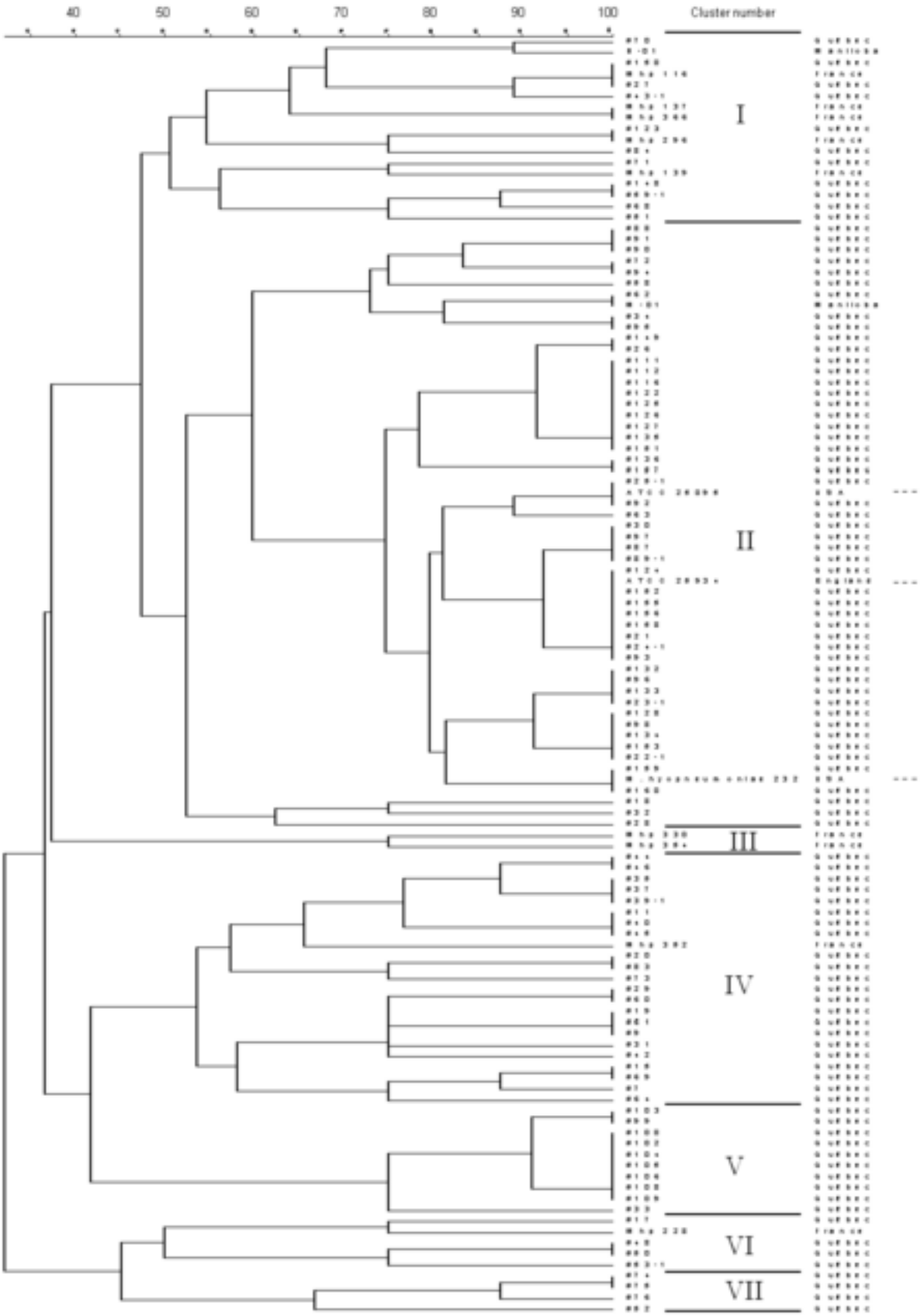

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